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Evaluation of *Trypanosama evansi* prevalence and risk factors in the one-humped camels (*Camelus dromedarius*) of the north-east of Iran by a real-time PCR test

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ABSTRACT

The Surra caused by *Trypanosoma evansi* (*T. evansi*) is an economically damaging disease of livestock including camels, horses, and buffaloes. The disease is transmitted by arthropod flies belonging to family tabanidae. The clinical signs of affected animals include recurrent fever, progressive anemia, cachexia, edema, and abortion. In order to determine the point prevalence of Surra in the camel population of north-east of Iran, 152 blood samples from one-humped camels were collected by multiple cluster sampling methods from three provinces, namely, Razavi Khorasan (R.Kh.), Northern Khorasan (N.Kh.), and Southern Khorasan (S.Kh.). The nucleic acid extracted from the buffy coat of each blood sample was analyzed by SYBR green real-time PCR test for the detection of *T. evansi* in the blood samples.

T. evansi was detected in 10 out of 152 camel blood samples (6.5%) with a prevalence rate of 8.6, 9.3, and 1.4 percent in R.Kh., N.Kh., and S.Kh. provinces, respectively. The prevalence of the disease decreased from north to south in the Khorasan provinces. Multivariate logistic regression analyses showed that among risk factors influencing Surra in the camel population, location was the most remarkable risk factor. Different geographical conditions, climate change, and the amount of raining can be considered as the factors affecting Surra vector population from north to south, resulting in a decrease in the rate of the prevalence of Surra from north to south.

1. Introduction

T. evansi is the most important and common protozoan parasite in camels and can cause camel trypanosomosis or Surra in Iran. Surra has a wide host range and the most important host species in different geographical regions varies (Juyal et al., 2005). The major host species in Africa are camels while the horse is mostly affected in the Center and South America. In Asia, very different hosts like cattle, buffalo, and pigs are mainly affected (Getachew, 2005; Basaznew et al., 2012; Diallo et al., 2018; Parashar et al., 2018). *T. evansi* is a vector-borne disease that is transmitted mechanically by haematophagus flies, like horseflies (*Tabanus*), and stable flies (*Stomoxys*) (Singla et al., 2015). In some countries, the incidence of Surra increases significantly during the rainy season when biting fly populations greatly increase (Dhami et al., 1999; OIE, 2013). Both acute and chronic forms of Surra are found in affected animals. Fever, anorexia, significant generalized edema, and finally death are seen in affected camels; the chronic form of Surra is manifested in the form of continuous body weight loss, intermittent fever, general muscular atrophy, anemia, and abdominal edema. The affected camels may be characterized by sweet odor because of an increase in the urinary ketones. *T. evansi* infection is commonly seen in the chronic form and may be associated with secondary infection and poor immune response to vaccination caused by an immune-suppression effect of the disease (Abdel-Rady, 2008; Gupta et al., 2009; Singla et al., 2009).

In Iran with semi-dry climate, plant coverage in half of the pastures is poor, so the camel husbandry has proven to be more and more important over the course of time and, gradually, more attention has been paid to the camel diseases in Iran. The purposes of the current study were to determine the prevalence and evaluate the risk factors associated with Surra in camels in the north-east of Iran. To achieve this objective, Real-time PCR assay was performed for the detection of the *T. evansi* in the camel blood samples. The data on sex, location, and age of sampled camels were recorded and analyzed.

Although different methods are available for detecting *T.evansi*, it has been emphasized that PCR is the most sensitive technique for the detection of the infection (OIE, 2012). Thus, for the first time in Iran,

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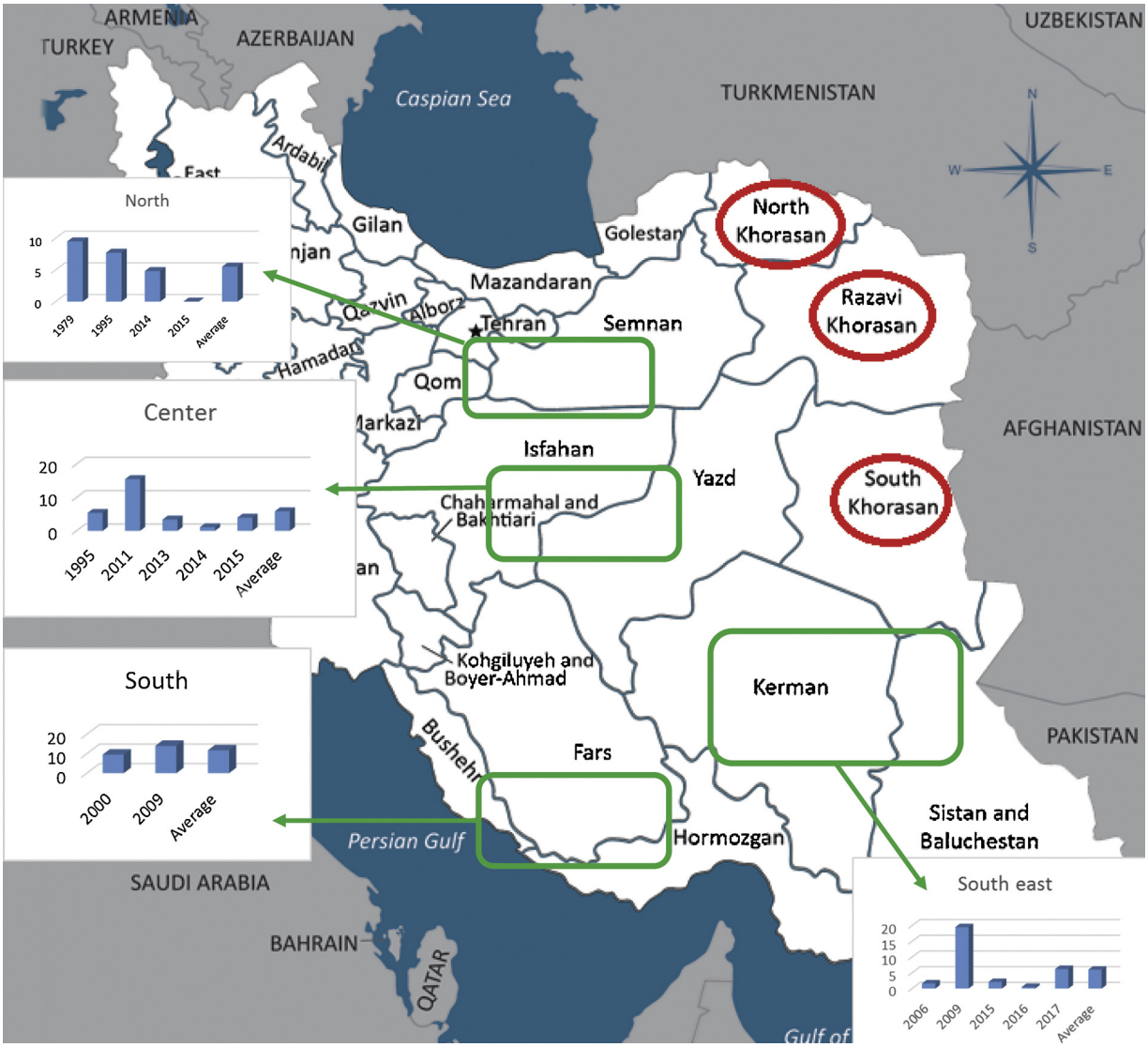

Fig. 1. The map of Iran. Study area provinces in the present study are circled. Study areas of previous studies and their results are in the rectangles. Prevalence rate (%) and the year of study are shown in Y and X axes, respectively.

we conducted a real-time PCR test (Konnai et al., 2009; Sharma et al., 2012) to detect the chronic cases of Surra in apparently healthy camels.

2. Material and methods

2.1. Study area and sampling

This study was conducted in three provinces of Iran, i.e. Razavi Khorasan (R.Kh.), Northern Khorasan (N.Kh.), and Southern Khorasan (S.Kh.), which are located at 55° 17 to 61° 15E and 30° 24 to 38°17N in the north-east of Iran. Northern Khorasan (N.Kh.), Razavi Khorasan (R.Kh.), and Southern Khorasan (S.Kh.) are located from north to south, respectively (Fig. 1).

The adequate number of samples was decided by this equation: $n = Z^2 * P_{exp} (1–P_{exp})/d^2$, where n is the sample size, P_{exp} is the expected prevalence, and d is the desired absolute precision. Z_{α} is the normal deviate (1.96) at 95% confidence level, P is the estimated prevalence, $q = 1 - P$, and d is the precision of the estimate. When P, 0.11, and d, 0.05, were added to the equation, a sample size of 150 was determined (Thrusfield, 2007).

The cluster random sampling strategy was recruited. Each cluster contained 11 randomly-selected counties; at least 11 camels from each cluster were sampled (Thrusfield, 2007). 10 ml of whole blood in EDTA (anti-coagulant) containing tube was collected from 152 apparently healthy camels sampled from three Khorasan provinces. The sample was labeled with the date of collection, animal number, sex, age, and area. Blood samples were transported to a laboratory in cold condition. In order to separate the buffy coats, the blood samples were centrifuged 1,000 g for 10 min in a swing-out rotor. Then, the buffy coats were collected and stored in −70 °C freezer until nucleic acid extraction.

2.2. Real-time PCR assay

2.2.1. Nucleic acid extraction

The nucleic acid of blood samples was extracted using high pure PCR template preparation kit manufactured by Roche®. The extracted DNA samples were electrophoresed on 1.5% agarose gel in order to check their quality. Also, the quantities of extracted DNA were measured by a NanoDrop spectrophotometer (Biotech, USA).

2.2.2. Real-time PCR and melting curve analysis

SYBR green real-time PCR test was utilized for the detection of *T. evansi* parasite. Real-time PCR was performed according to a published paper (Konnai et al., 2009) with some modifications. For each sample,

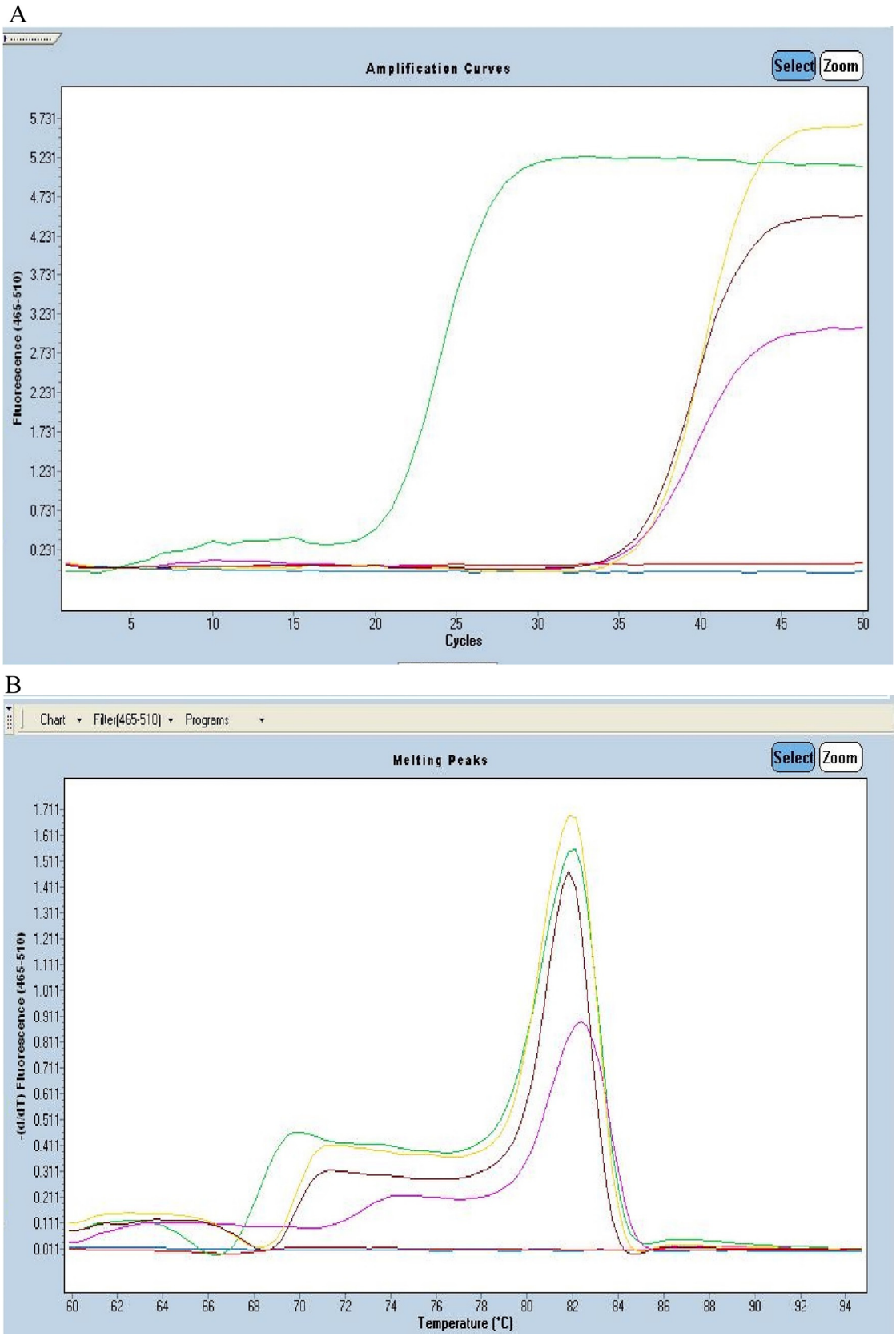

Fig. 2. The graphs of Lightcycler 480 thermocycler. A) Positive and negative results of SYBR Green Real-Time PCR test. B) The melting peak of positive and negative samples of *T. evansi* Real-Time PCR test.

Table 1
Prevalence of Surra with respect to sex, age, district, and province for the camel population of a wide area of north-east of Iran, bordered by Afghanistan, Pakistan, and Turkmenistan; the P value of Chi-square test is shown and $P < 0.25$ were then used for multivariate logistic regression.

Parameter	Tested camel (+ve)	p value
Sex		
Male	26 (3)	0.233
Female	126 (7)	
Age (year)		
= < 3	52 (1)	0.087
> 3	100 (9)	
District		
Mangali	14 (5)	0.000
Chehel dokhtaran	14 (0)	
Tapeh salam	14 (4)	
Sabzehvar	14 (0)	
Ghochan	14 (0)	
Robat e sang	13 (0)	
Boshroyeh	14 (0)	
Kani mani	13 (0)	
Birjand	18 (0)	
Sarayan	13 (1)	
Nehbandan	11 (0)	
Provinces		
Northern Khorasan	39 (4)	0.12
Razavi Khorasan	53 (5)	
Southern Khorasan	60 (1)	

Table 2
A multivariate logistic regression model showing factors influencing the risk of Surra in camel population in a wide area of north-east Iran.

Independent variable	B (SE)	OR (95 % CI)	*P*
Location (Province name)			
Southern Khorasan	1, reference	–	–
Razavi Khorasan	1.46 (1.13)	4.31	0.198
Northern Khorasan	2.03 (1.11)	7.63	0.069

the total volume of 20µl was mixed containing 10 µl of 2X master mix, 5.2 µl of DNase free water, 0.4 µl of each primer (10 µM), and 4 µl of DNA template. DNase-free water and DNA of a microscopically confirmed positive sample were used in every run of the real-time PCR as no-template control (NTC) and positive control, respectively. The thermal amplification protocol was 5 min at 95 °C and 40 cycles of 95 °C for 10 s, 62 °C for 30 s, and 72 °C for 20 s. After cyclic amplification was completed, melting curve from 60 °C to 95 °C was determined and analyzed. Real-time PCR was performed using Lightcycler® 480 (Roche, Germany) thermocycler instrument and Lightcycler® 480 SYBR green I master (Roche, Germany).

2.3. Statistical analysis

Significance testing of each independent variable was performed by running a Chi-square test. Chi-square test analysis was first conducted to identify which independent variable(s) could be appropriate for multivariate logistic regression modeling. Predictors with $P < 0.25$ were placed into a multivariate logistic regression model. A forward step-wise approach was used to identify explanatory variables, related to the Surra positivity. All statistical analyses were performed using SPSS statistical software version 21 (SPSS Inc., Chicago). $P < 0.05$ was considered as significant.

3. Result

3.1. Real-time PCR

After quality control, DNA samples were subjected to SYBR Green Real-Time PCR test to detect the *T. evansi*. One of the Real-Time PCR graphs (Fig. 2A) show that the positive samples depict good sigmoid curves while the negative ones have no upward rises. Also, melting curve analysis showed that the positive results were associated to one desired PCR product. As it could be seen in Fig. 2B, there are melting peaks on 82 °C in only one position which shows the accuracy and specificity of the test.

3.2. Prevalence of T. evansi in the camels of the north east of Iran

From 152 blood samples that were tested for the detection of *T. evansi* by SYBR green real-time PCR test, 10 positive samples were detected. Thus, the prevalence of Surra disease in the camels of north-east of Iran would be 6.5%. The disease prevalence rates in Razavi Khorasan (R.Kh.), Northern Khorasan (N.Kh.), and Southern Khorasan (S.Kh.) were 10.25, 9.43, and 1.6%, respectively. The descriptive insight of Surra prevalence in relation to the age, sex, district, and location of the camel populations is shown in Table 1.

3.3. Risk factors of Surra in camels

Based on Chi-square analyses, sex, location, and age were found to be appropriate independent parameters for further multivariate logistic

Table 3
The prevalence of *T. evansi* infection in dromedary camels of different areas of Iran. SE refers to the smear examination.

Geographic area	Sampling province(s)	Animal number	*T. evansi* Prevalence (%)	Test method(s)	Reference and year
Sub-North	Tehran	127	9.5	SE	Badamchi (1979)
	Tehran	196	7.7	SE	Rahbari and Bazargani (1995)
	Semnan	21	4.8	SE	Ahmadi Hamedani et.al., (2014)
	Tehran	100	0.0	SE	Majidi Rad et al. (2015)
North east	Razavi Khorasan	262	0.58	SE	Borji et.al. (2009)
Center	Isfahan	37	5.4	SE	Mizan Zadeh (1995)
	Yazd	110	15.5	SE	Sazmand et al. (2011)
	Yazd	117	3.4	SE + PCR	Pourjafar et al. (2013)
	Isfahan	278	1.1	PCR	Mehrabiyan et al. (2014)
	Isfahan & Yazd	227	3.96	SE	Karimi et al. (2015)
South	Bushehr	333	9.5	SE	Zarif Fard and Hashemi Fesharaki (2000)
	Fars	285	14	SE	Moghaddar and Dianat pour (2009)
South east	Kerman	60	1.6	SE	Radfar et al. (2006)
	Sistan	113	19.5	SE	Ranjbar Bahadori and Afshari Moghaddam (2009)
	Kerman	95	2.1	SE + PCR	Khosravi et al. (2015)
	Kerman & Sistan-va-Bloochestan	200	0.5	SE + PCR	Sazmand et al. (2016)
	Sistan		6.2	SE + PCR	Zangooyi (2017)
South west	Khuzestan		19	SE + PCR	Zakian et al. (2017)

regression analyses ($P < 0.25$). Multivariate logistic regression models did not show sex, location or age to be significantly correlated with the Surra at the individual level in camels ($P < 0.05$), but among the tested risk factors influencing Surra in camel population, location was the most remarkable risk factor. By moving from south (S.Kh.) to north (N.Kh.), the chance of being infected with *T. evansi* increased and the risk of getting Surra in N.Kh. was 7.63-fold to being infected in S.Kh. (Odds ratio (OR) = 7.63, $P = 0.06$). Also, in R.Kh., the middle province, the risk of getting Surra was 4.31-fold to being infected in Kh. J. (Table 2).

4. Discussion

In this study, the real-time PCR (a rapid, sensitive and specific diagnostic tool that has been widely used for pathogens) was recruited for the detection of Surra in the camels of north-east (three provinces) of Iran. In the previous studies of Surra prevalence in Iran, different results were obtained (Table 3). First, in some studies (Borji et al., 2009; Radfar et al., 2006; Ranjbar Bahadori and Afshari Moghaddam, 2009), the samples were collected from slaughter houses where generally more infections may be detected because the Surra is a chronic disease. Secondly, trypanosome infections are often misdiagnosed due to the low sensitivity and specificity of serological and parasitological tests. In addition, pathognomonic clinical and postmortem changes are lacking. Hence, there is a need to include PCR as part of the strategy to improve the reliability of the diagnosis (Beltran et al., 2004; Tehseen et al., 2017). This study documented the use of Real-time PCR as a diagnostic tool for detecting *T. evansi* in the camels. The major advantage of this method is that it enables the researchers to quantify pathogen in a given sample. Although this merit was not utilized in this study, it is suggested that in the future studies, the researchers correlate the number of *T. evansi* in blood to find the disease phase of Surra in camels. PCR method for studying Surra in camels have frequently been used and the genes of *T.evansi* mostly targeted in these PCRs were *RoTat1.2*, *ITS-1,* and *ESAG 6/7* (Salim et al., 2011; Barghash et al., 2014; Khosravi et al., 2015; Sazmand et al., 2016; Zangooyi, 2017; Zakian et al., 2017). To the best of our knowledge, this is the first use of real-time PCR for the epidemiological studies of *T. evansi* in camels in Iran and the second worldwide. Only one study conducted real-time PCR for the detection of Surra in camels previously (Yousef et al., 2010).

Eighteen previous studies on the prevalence of Surra in different areas of Iran have been conducted since 40 years ago. South-eastern and central parts of Iran were more investigated for Surra and the reason for such focus on these areas is that these parts of Iran represent the most camel rearing areas in Iran. In some studies, a relationship was found between higher ages and Surra infection in camels of Iran (Khosravi et al., 2015, Moghaddar and Dianatpour, 2009). The season was another factor that was found to affect the spread of Surra in Iran (Zarif Fard and Hashemi Fesharaki, 2000). Zarif fard et al. found that in winter and spring, in comparison with summer and fall, Surra spread more rapidly in one of the southern provinces, Bushehr. Due to the hot and humid weather of this province during summer and autumn, vector activity declines during these seasons. Therefore, it seems that the effect of season may be due to the vector population in different seasons. Other studies of Surra in Iran did not consider different risk factors or they did not found a significant relationship caused by age, gender, and/or season (Karimi et al., 2015); a finding which is consistent with our results.

In the present study, the prevalence of Surra in the camels of the north-east of Iran was measured as 6.5% that is higher than the number reported in the only previous study in this area. In that study, the smear examination of the blood samples of slaughtered camels was used and the *T. evansi* prevalence was found to be 0.58% (Borji et al., 2009). As real-time PCR method is much more sensitive than smear examination (SE) (Yousef et al., 2010), we cannot exactly compare the findings of the present study and that of Borji's study and conclude that Surra prevalence increased in north-east of Iran. In other Surra prevalence studies in Iran, different results, ranging from 0 to %19.5 have been reported (Table 3). Surra prevalence average of the previous studies in Iran is 6.9% which compared with our result (6.5%) does not show a very different prevalence rate. If the averages of Surra prevalence in different regions of Iran are compared, we can have a clearer and broader vision of Surra in Iran. The averages of Surra prevalence in central (5 studies), south-eastern (5 studies), sub-northern (4 studies), southern (2 studies), and south-western (1 studies) regions of Iran were 5.87%, 5.98%, 5.5%, 11.75%, 19%, respectively (Table 3). Comparing our results with results reported for other regions of Iran, it seems that the Surra prevalence in the north-east of Iran is slightly more than that of central, south-eastern, and sub-northern regions of Iran, but dramatically lesser than that of south-western and southern regions. The reason for approximately two to three folds of increase of prevalence in these regions can be attributed to the point that the studies on Surra in south-western and southern regions of Iran were reported with regard to, or at the time of, outbreaks of Surra in these regions (Zakian et al., 2017; Zarif Fard and Hashemi Fesharaki, 2000).

Our results showed that N.Kh. (10.25%), R.Kh. (9.43%), and S.Kh. (1.6%) had the most to the least prevalence of Surra, respectively. The logistic regression model showed that the location (province) was a factor influencing the risk of Surra in camel population in a wide part of Iran. There is a considerable topological difference in the regions examined for this study, ranging from the northern part (N.Kh. and part of R.Kh.) covered with high mountain chains (Binalud Mountains) to the southern part (S.Kh.) with fewer scattered mountains and deserts. The climate of these provinces was totally different, from humid mountains to dry desert. The annual amount of rain in the long-term period in N.Kh, R.Kh. and S.Kh. are 262, 203, and 113 mm, respectively, indicating the decrease of humidity from the northern province to the southern one. In OIE Technical Disease Card for Trypanosoma evansi infection (Surra), the relationship between rainy seasons and a significant increase in Surra is highlighted (OIE, 2013). Different geographical conditions, climate change, and the amount of raining can be considered as the factors affecting Surra vector population from north to the south of Khorasan provinces. This finding is consistent with the results reported by Hagos et al. (2009) who studied Surra in camels of two areas with different ecological characteristics (wet and dry) in Ethiopia. They found that camels in riverine areas of Dello-Mena were more prone to infection than herds in the non-riverine areas of Sawena (Hagos et al., 2009). Finally, it could be concluded that the decrease of the prevalence of the Surra from north to south of Khorasan provinces could be due to the factors that cause the decline of the vector population. Further studies are needed to focus on Surra vector population in these regions and to evaluate other risk factors for planning future control programs.

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